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Analysis of chicken cytokine and chemokine gene expression following *Eimeria acervulina* and *Eimeria tenella* infections

Yeong Ho Hong [a], Hyun S. Lillehoj [a,*], Sung Hyen Lee [a], Rami A. Dalloul [a], Erik P. Lillehoj [b]

[a] *Animal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, United States Department of Agriculture, Building 1040, BARC-East, Beltsville, MD 20705, United States*
[b] *Department of Pediatrics, School of Medicine, University of Maryland, Baltimore, MD 21201, United States*

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Abstract

The expression levels of mRNA encoding a panel of 28 chicken cytokines and chemokines were quantified in intestinal lymphocytes following *Eimeria acervulina* and *Eimeria tenella* primary and secondary infections. Compared with uninfected controls, transcripts of the pro-inflammatory cytokines IFN-α, IL-1β, IL-6, and IL-17 were increased up to 2020-fold following primary infection. By contrast, following secondary infection by either microorganism, pro-inflammatory mRNAs levels were relatively unchanged (≤20-fold). Transcripts encoding the Th1 and Th1 regulatory cytokines IFN-γ, IL-2, IL-10, IL-12, IL-15, IL-16, and IL-18 were uniformly increased 14–2471-fold after *E. acervulina* primary infection, but either unchanged (IL-15, IL-16, IL-18), increased (IFN-γ, IL-10, IL-12), or decreased (IL-2) following *E. tenella* primary infection. Following secondary infections, Th1 cytokine mRNA levels were relatively unchanged, with the exception of IL-12 which was increased 1.5×10^5-fold after *E. acervulina* and decreased 5.1×10^4-fold after *E. tenella* infection. Transcripts for the Th2 or Th2 regulatory cytokines IL-3 and GM-CSF were increased up to 327-fold following primary or secondary infection with both parasites, while IL-4 and IL-13 mRNAs were decreased 25- to 2×10^5-fold after primary or secondary infection. The dynamics of chicken chemokine expression revealed modest changes (<100-fold) following primary or secondary infection except for lymphotactin. When lymphocyte subpopulations were similarly analyzed, IFN-γ, IL-2, IL-3, IL-15, and MIF were most highly increased in $TCR2^+$ cells following *E. acervulina* infection, while $TCR1^+$ cells only expressed high levels of IL-16 following *E. tenella* infection. In contrast, $CD4^+$ cells only expressed highest levels of IL-10 after *E. acervulina* infection, whereas these cells produced abundant transcripts for IFN-γ, IL-3, IL-15, and MIF after *E. tenella* infection. We conclude that coccidiosis induces a diverse and robust primary cytokine/chemokine response, but a more subdued secondary response.

Keywords: *Eimeria*; Cytokines; Chemokines; T lymphocytes; Quantitative RT-PCR

Abbreviations: DPI, days post-primary infection; DSI, days post-secondary infection; IELs, intraepithelial lymphocytes; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GM-CSF, granulocyte monocyte-colony stimulating factor; iNOS, inducible nitric oxide synthase; MIF, migration inhibition factor; MIP-1β, macrophage inflammatory protein-1β; MyD88, myeloid differentiation factor 88; T-bet, T-box expressed in T cells; TGF-β4, transforming growth factor-β4; TRAF-5, TNF receptor-associated factor-5

* Corresponding author. Tel.: +1 301 504 8771; fax: +1 301 504 5103.
E-mail address: hlilleho@anri.barc.usda.gov (H.S. Lillehoj).

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1. Introduction

Parasite infection of the intestinal tract of domestic food animals is a major veterinary health problem worldwide. Avian coccidiosis is an intestinal infection caused by apicomplexan protozoa belonging to at least seven different species of *Eimeria.* A variety of strategies are used to reduce the negative impact of coccidiosis, including prophylaxis with anti-coccidial drugs, selection of disease resistant chicken strains, and augmentation of immunity (Lillehoj, 1994; Caron et al., 1997; Pinard-Van Der Laan et al., 1998; Yun et al., 2000c). None of these methods, however, are without drawbacks. Coccidiosis has been estimated to be responsible for monetary losses greater than $3 billion annually (Williams, 1999). Approximately 80% of this cost is due to the direct effects on mortality, weight gain and feed conversion, and 20% is due to the cost of chemoprophylaxis.

Vaccination against coccidiosis relies on the fact that acquired immunity develops following exposure to live *Eimeria* parasites. Chickens that have been vaccinated or naturally infected with coccidian parasites produce specific immunity mediated by T lymphocytes, and cell-mediated immune responses are the major factors conferring resistance to coccidiosis. The list of vaccines against *Eimeria* includes live virulent organisms, live attenuated strains of parasites, non-infective parasite derivatives, and genetically engineered subunits vaccines (Lillehoj and Lillehoj, 2000; Yun et al., 2000b; Dalloul and Lillehoj, 2006). In general, live vaccines are the most efficient at producing long-lasting protective immunity, in large part because they most closely replicate natural intestinal infection. Identification of the molecular profile of early host cellular immune responses provides a clue of the events that occur following infection with *Eimeria.* Particularly relevant in this regard is the observation that natural infection or vaccination with live parasites induces the production of a plethora of chicken cytokines and chemokines critical for mounting protective immunity (Kaiser et al., 2005).

Until recently, relatively few chicken cytokines had been described. The first of these to be isolated and characterized included interferon (IFN)-γ, interleukin (IL)-2, and transforming growth factor (TGF). However, with the advent of the chicken genome project, a number of chicken cytokine and chemokine genes have been discovered (Hughes and Bumstead, 2000; Schneider et al., 2000; Sick et al., 2000; Min and Lillehoj, 2002; Avery et al., 2004; Degen et al., 2004; Min and Lillehoj, 2004; Rothwell et al., 2004; Kaiser et al., 2005; Read et al., 2005; Wang et al., 2005; Hong et al., 2006). Cloning of these genes has led to the development of a comprehensive array of reagents for investigating avian innate and acquired immune responses at the molecular and cellular levels that was not possible only a few years ago (Swaggerty et al., 2004). In the current study, we analyzed the expression of a panel of genes encoding pro-inflammatory, Th1, and Th2 cytokines, and chemokines, following experimental infection of chickens with *Eimeria acervulina* or *Eimeria tenella.* Along with *Eimeria maxima*, these three *Eimeria* species are responsible for the majority of coccidiosis in commercial production facilities. Our goal was to identify those molecules that were either up- or down-regulated during coccidiosis as candidates for future immunomodulation studies.

2. Materials and methods

2.1. Animals, parasites infection, and isolation of IELs

Fertilized eggs of specific pathogen-free chickens were obtained from SPAFAS (Charles River Laboratories, Preston, CT) and hatched at the Animal and Natural Resources Institute, USDA (Beltsville, MD). The chicks were kept in wire cages with feed and water provided *ad libitum*, and orally inoculated with 1×10^4 sporulated oocysts of *E. acervulina* or *E. tenella* at 3 weeks of age as described (Min et al., 2001). Secondary infection with 2×10^4 oocysts was performed at day 14 post-primary infection. At 1 day intervals following primary or secondary infections, the intestinal duodenum and ceca were removed from five chickens, cut longitudinally, and washed three times with ice-cold Hank's balanced salt solution (HBSS) containing 100 units/ml of penicillin and 100 μg/ml of streptomycin (Sigma, St. Louis, MO). The mucosal layer was carefully scraped away using a surgical scalpel, the tissue was washed several times with HBSS containing 0.5 mM EDTA and 5% fetal calf serum (FCS) and incubated for 20 min at 37 °C with constant swirling. Cells released into the supernatant were pooled, passed through nylon wool (Robbins Scientific, Sunnyvale, CA) to remove dead cells and cell aggregates and washed twice with HBSS. All experiments were approved by the Animal and Natural Resources Institute IACUC.

2.2. cDNA synthesis

Total RNA was extracted from IELs using TRIzol (Invitrogen, Carlsbad, CA) as described (Min et al., 2001). Five micrograms of total RNA were treated with 1.0 unit of DNase I and 1.0 μl of 10× reaction buffer (Sigma), incubated for 15 min at room temperature,

1.0 μl of stop solution was added to inactivate DNase I, and the mixture was heated at 70 °C for 10 min. RNA was reverse transcribed using StrataScript first-strand synthesis system (Stratagene, La Jolla, CA) according to the manufacturer's recommendations. Briefly, 5.0 μg of RNA were combined with 10× first strand buffer, 1.0 μl of oligo(dT) primer (5 μg/μl), 0.8 μ1 of dNTP mix (25 mM of each dNTP), and RNase-free water to total volume 19 μl. The mixture was incubated at 65 °C for 5 min, cooled to room temperature, 50 units of StrataScript reverse transcriptase was added, the mixture was incubated at 42 °C for 1 h, and the reaction stopped by heating at 70 °C for 5 min.

2.3. Quantitative RT-PCR

Oligonucleotide primers for chicken cytokines, chemokines, and GAPDH control were designed based upon sequences available from public databases (Table 1). Amplification and detection were carried out using equivalent amounts of total RNA from IELs using the Mx3000P system and Brilliant SYBR Green QPCR master mix (Stratagene). Standard curves were generated using $\log_{10}$ diluted cDNA from pooled infected and non-infected total RNA. To normalize individual replicates, the logarithmic scaled raw data unit cycle threshold (CT) was transformed into the linear unit of normalized expressions and then calculating means and S.E. for the references (CTreference, mean and $S.E._{CTreference,\ mean}$) and individual targets (CTtarget, mean and $S.E._{CTtarget,\ mean}$), followed by determination of a mean normalized expression (MNE) using the Q-gene program (Muller et al., 2002). The GAPDH gene was chosen as a normalizer because this gene is less abundant than 28S, it will be helpful to normalize a rare target gene accurately and no differences in expression patterns compared to 28S for IFN-γ, MyD88, and MIP-1β (data not shown). Each analysis was performed in triplicate. To correct for differences between RNA levels between samples within the experiment, the difference factor for each sample was calculated by dividing the mean threshold cycle (C_t) values for GAPDH from all samples in that experiment.

Table 1
Sequence of the primers used in real-time quantitative RT-PCR

RNA target	Primer sequences		Size for PCR product (bp)	Accession no.
	Forward	Reverse		
GAPDH	5′-GGTGGTGCTAAGCGTGTTAT-3′	5′-ACCTCTGTCATCTCTCCACA-3′	264	K01458
IFN-γ	5′-AGCTGACGGTGGACCTATTATT-3′	5′-GGCTTTGCGCTGGATTC-3′	259	Y07922
IFN-α	5′-GACATCCTTCAGCATCTCTTCA-3′	5′-AGGCGCTGTAATCGTTGTCT-3′	238	AB021154
IL-1β	5′-TGGGCATCAAGGGCTACA-3′	5′-TCGGGTTGGTTGGTGATG-3′	244	Y15006
IL-2	5′-TCTGGGACCACTGTATGCTCT-3′	5′-ACACCAGTGGGAAACAGTATCA-3′	256	AF000631
IL-3	5′-CTCTGCCTGCTGCTGTCC-3′	5′-TTATCTGCTTTTTGCTGCTTTC-3′	238	AJ621740
IL-4	5′-ACCCAGGGCATCCAGAAG-3′	5′-CAGTGCCGGCAAGAAGTT-3′	258	AJ621735
IL-6	5′-CAAGGTGACGGAGGAGGAC-3′	5′-TGGCGAGGAGGGATTTCT-3′	254	AJ309540
IL-8	5′-GGCTTGCTAGGGGAAATGA-3′	5′-AGCTGACTCTGACTAGGAAACTGT-3′	200	AJ009800
IL-10	5′-CGGGAGCTGAGGGTGAA-3′	5′-GTGAAGAAGCGGTGACAGC-3′	272	AJ621614
IL-12p40	5′-AGACTCCAATGGGCAAATGA-3′	5′-CTCTTCGGCAAATGGACAGT-3′	274	NM_213571
IL-13	5′-CCAGGGCATCCAGAAGC-3′	5′-CAGTGCCGGCAAGAAGTT-3′	256	AJ621735
IL-15	5′-TCTGTTCTTCTGTTCTGAGTGATG-3′	5′-AGTGATTTGCTTCTGTCTTTGGTA-3′	243	AF139097
IL-16	5′-TCCCTCTGCAAAATGGTCA-3′	5′-TCGCGATCTCAGGTTGTGT-3′	271	AJ508678
IL-17	5′-CTCCGATCCCTTATTCTCCTC-3′	5′-AAGCGGTTGTGGTCCTCAT-3′	292	AJ493595
IL-18	5′-GGAATGCGATGCCTTTTG-3′	5′-ATTTTCCCATGCTCTTTCTCA-3′	264	AJ277865
TGF-β4	5′-CGGGACGGATGAGAAGAAC-3′	5′-CGGCCCACGTAGTAAATGAT-3′	258	M31160
GM-CSF	5′-CGCCCACCACAACATACTC-3′	5′-ACGATTCCGCTTTCTTCCT-3′	202	AJ621740
MIF	5′-GCAGCCTCTACAGCATTGG-3′	5′-TCTAACGGGCAGCACGAG-3′	229	M95776
K60	5′-ATTTCCTCCTGCCTCCTACA-3′	5′-GTGACTGGCAAAAATGACTCC-3′	228	AF277660
K203	5′-ACCACGAGCTCCTGACACA-3′	5′-TTAAATGCCCTCCCTACCAC-3′	300	Y18692
MIP-1β	5′-GTGCCCTCATGCTGGTGT-3′	5′-GGTTGGATGCGGATTATTTC-3′	285	L34553
Lymphotactin	5′-GGATTTAAGGGTGAACAGTAGATG-3′	5′-TAGAAATAGAAAGCCCGAGGAT-3′	254	AF006742
SDF-1	5′-AACAAGTAAGGCAAGAGACAAATG-3′	5′-GGGACCCCAGCACAGATAC-3′	264	AY451855
TRAF-5	5′-TGATTATCCCATGCCTTGTCT-3′	5′-CTCTGCTAGCTGCTGGATTTTA-3′	283	NM_204219
MyD88	5′-TCC CGG CGG TAG ACA GC-3′	5′-ACG ACC ACC ATC CTC CGA CAC CTT-3′	274	NM_001030962
iNOS	5′-TGGGTGGAAGCCGAAATA-3′	5′-GTACCAGCCGTTGAAAGGAC-3′	241	U46504
T-bet	5′-GGG AAC CGC CTC TAC CTG-3′	5′-AGTGATGTCGGCGTTCTGG-3′	288	CB016768

2.4. Magnetic cell separation and flow cytometric analysis

IELs were isolated from *E. acervulina*- or *E. tenella*-infected chickens at days 3 and 4 or days 7 and 8 post-primary infection and sorted into marker-specific fractions ($CD4^+$, $CD8^+$, $TCR1^+$, and $TCR2^+$) by using the magnetic BD IMag cell separation system (BD Biosciences Pharmingen, San Jose, CA) as previously described (Hong et al., 2006). Depleted cells were resuspended in HBSS without phenol red supplemented with 3% FCS and 0.01% sodium azide (FCA buffer). One hundred microliters (1×10^6 cells) were incubated for 40 min on ice with 100 μl of mouse monoclonal antibodies (mAbs) specific for CD4, CD8, TCR1, or TCR2 (Lillehoj et al., 1988). Following incubation, the

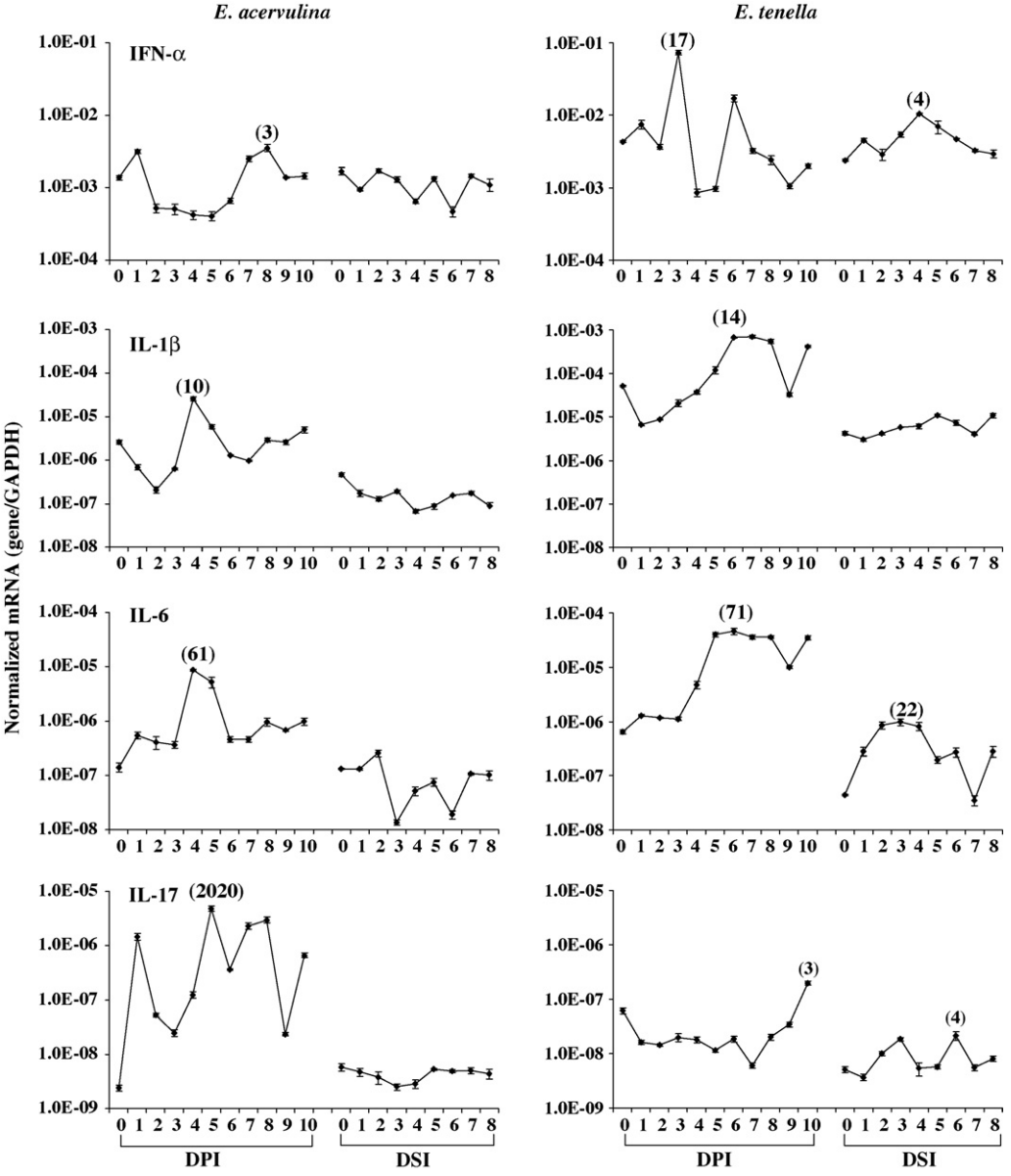

Fig. 1. Pro-inflammatory cytokine mRNA levels in intestinal IELs after *Eimeria acervulina* or *Eimeria tenella* primary and secondary infections. Chickens were non-infected (day 0) or orally infected with 1×10^4 oocysts of *E. acervulina* or *E. tenella* on day 0 and reinfected with 2×10^4 oocysts on day 14 post-primary infection. IELs were isolated from the jejunum and cecum at the indicated times post-infection and mRNA levels were determined by quantitative RT-PCR. Data are expressed as normalized mRNA levels to GAPDH mRNA levels of triplicate determinations with pooled samples from five chickens. Ratios between gene expression values at particular days when compared with non-infected values (0 day at each DPI and DSI) are indicated in parentheses and are statistically significant at least level $p < 0.01$. DPI, days post-primary infection; DSI, days post-secondary infection.

cells were washed two times with FCA buffer, incubated with 50 μl of fluorescein isothiocyanate FITC-labeled goat anti-mouse IgG secondary antibody (Sigma) for 30 min on ice, the cells washed two times, resuspended in 2.0 ml, and analyzed with an Epics model XL flow cytometer (Coulter, Miami, FL).

2.5. Statistical analysis

Mean ± S.E. values for each group ($N = 3$) were calculated, differences between groups were analyzed by the Dunnet multiple comparison test using InStat® software (Graphpad, San Diego, CA), and considered significant at $p < 0.01$.

3. Results

3.1. Pro-inflammatory cytokine response during Eimeria infection

T cell inflammatory responses are the major immune reactions in chickens infected with *Eimeria*, and confer

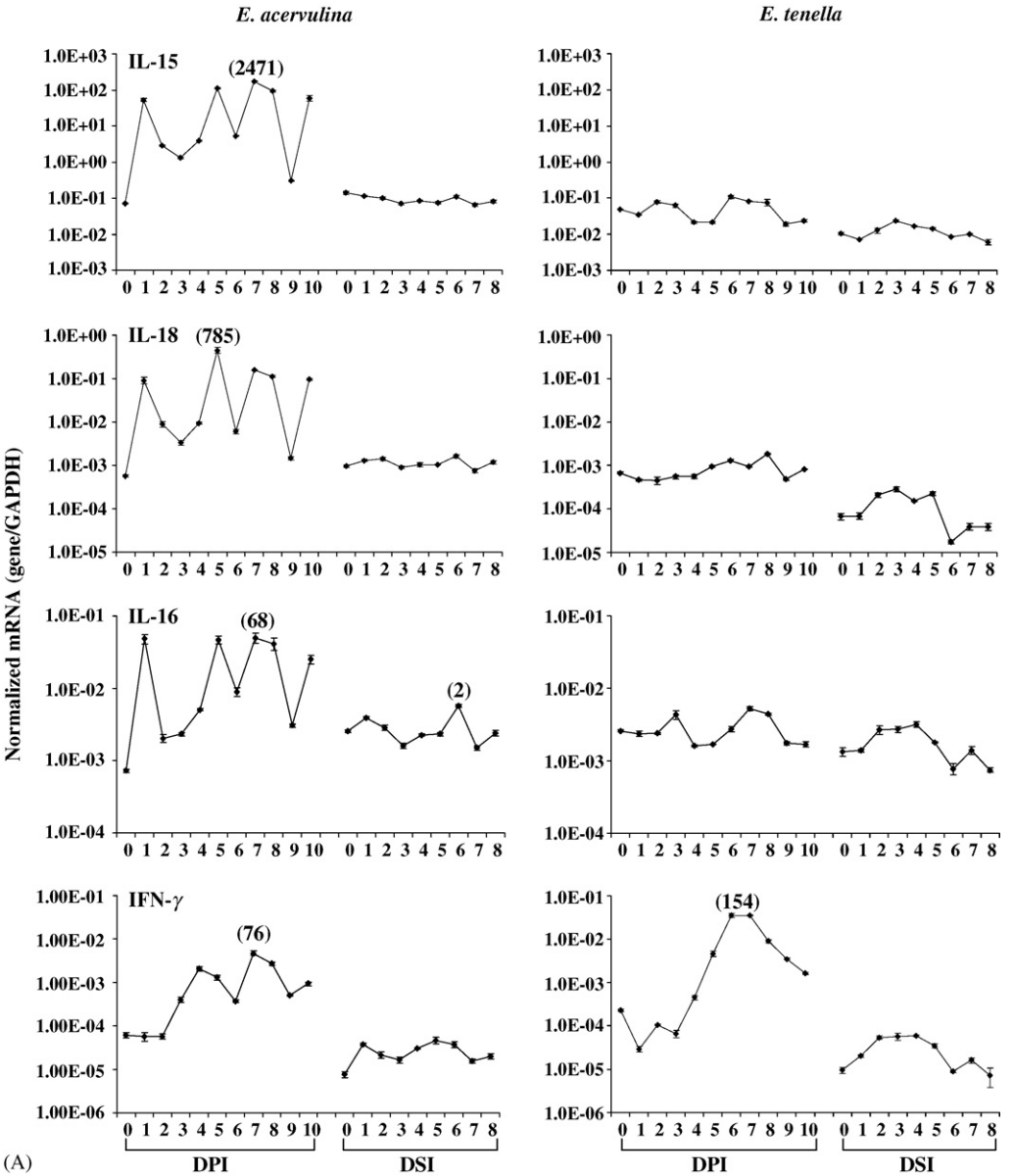

Fig. 2. Th1 and Th1 related cytokine mRNA levels in intestinal IELs after *E. acervulina* or *E. tenella* primary and secondary infections. Chickens were infected with *E. acervulina* and *E. tenella* and mRNA levels determined as described in Fig. 1.

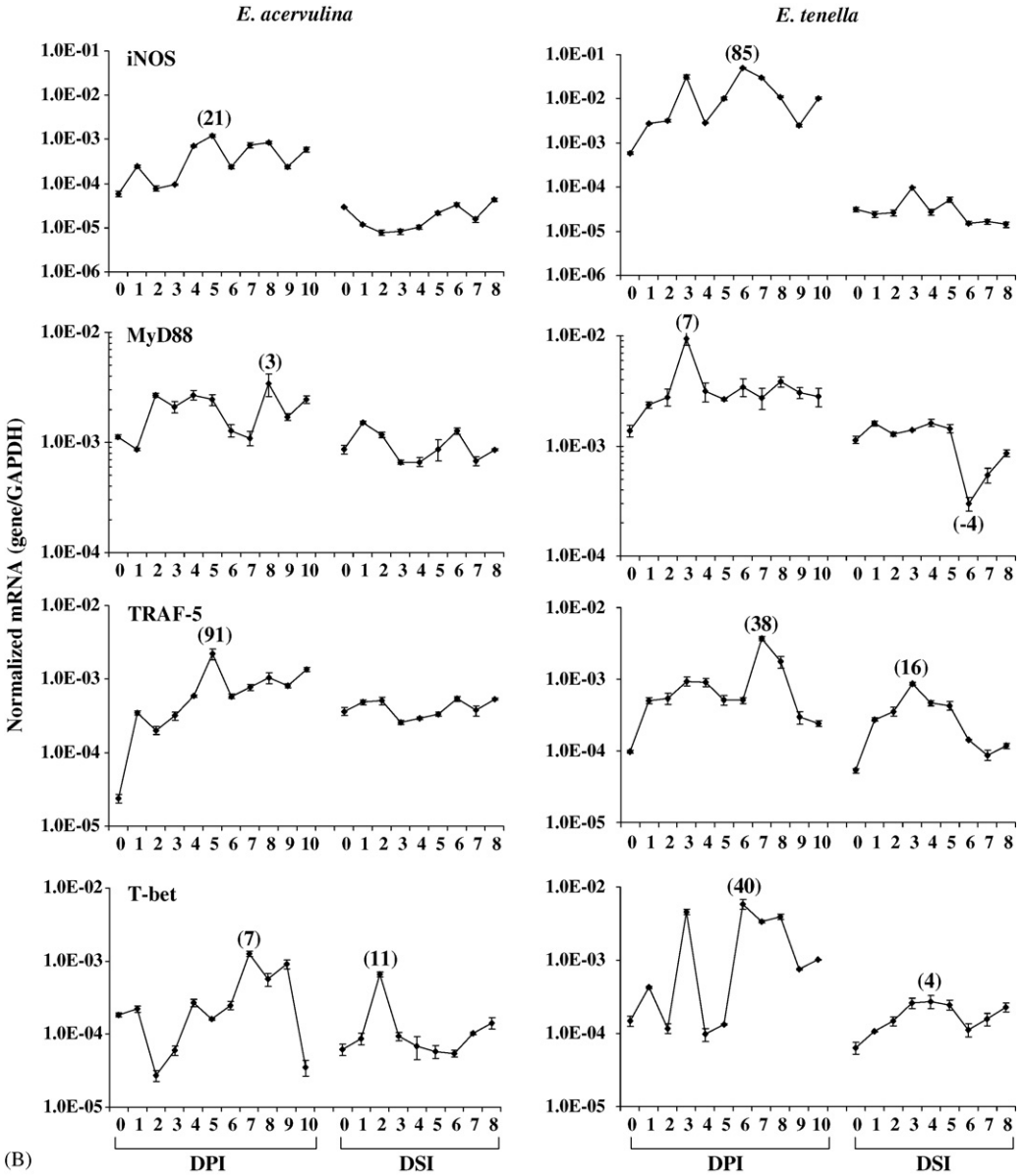

Fig. 2. (*Continued*).

protection against reinfection (Lillehoj and Trout, 1996; Lillehoj, 1998; Lillehoj and Lillehoj, 2000; Lillehoj et al., 2004). Our previous studies demonstrated that several pro- and anti-inflammatory cytokines were produced in response to experimental *Eimeria* infections (Lillehoj and Choi, 1998; Min et al., 2001). However, detailed kinetics of expression for these cytokines and others that have since been described, have not been determined. Therefore, we determined the expression levels of intestinal IEL gene transcripts encoding IFN-α, IL-1β, IL-6, and IL-17 at daily intervals for 10 days following primary infection with *E. acervulina* or *E. tenella*, and for 8 days following secondary infection. As shown in Fig. 1, three of the cytokines (IL-1β, IL-6, and IL-17) showed significantly higher mRNA levels following primary inoculation with *E. acervulina*, and four of the cytokines (IFN-α, IL-1β, IL-6, and IL-17) were up-regulated after *E. tenella* infection compared with non-infected animals (day 0). The increased transcript levels ranged from 10- to 2020-fold for *E. acervulina* and 14–71-fold for *E. tenella*. In contrast, transcripts for all cytokines were little or slightly changed following secondary infection except for IL-6 (22-fold) in *E. tenella*-infected animals.

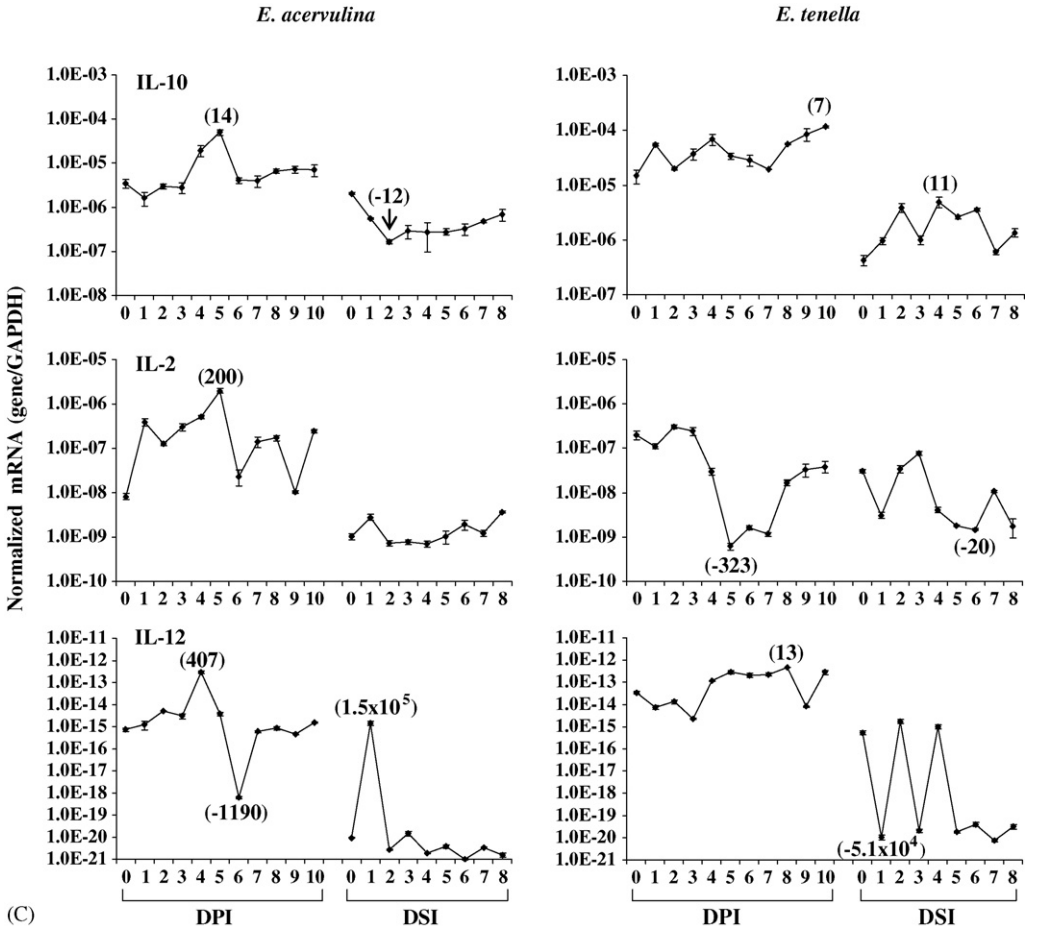

Fig. 2. (*Continued*).

3.2. Th1 and its regulatory cytokine response during Eimeria infection

Analysis of intestinal IEL transcripts encoding the Th1 and its regulatory cytokines IFN-γ, IL-2, IL-10, IL-12, IL-15, IL-16, and IL-18, as well as for MyD88, TRAF-5, iNOS, and T-bet, revealed that IFN-γ, IL-2, IL-12, IL-15, IL-16, IL-18, and TRAF-5 were increased >50-fold following *E. acervulina* primary infection, but <50-fold after *E. tenella* primary infection except for IFN-γ and iNOS (Fig. 2). In contrast, following secondary infection transcripts for TRAF-5, MyD88, T-bet, IL-2, IL-10, and IL-12 were altered, being increased by 11-fold to 1.5×10^5-fold in TRAF-5, T-bet, IL-10, and IL-12 and decreased 4-fold to 5.1×10^4-fold in MyD88, IL-2, IL-10, and IL-12. Especially, the only other down-regulated transcript was IL-2 during primary *E. tenella* infection, consistent with previous observations following *E. maxima* infection (Y.H. Hong, unpublished data).

3.3. Th2 and its regulatory cytokine response during Eimeria infection

Th2 lymphocytes produce cytokines that are important for the generation of type 2 immunity. Following primary *E. acervulina* infection, Th2 and regulatory cytokines were either slightly changed (IL-4, TGF-β4), moderately increased (GM-CSF), drastically increased (IL-3), or drastically decreased (IL-13). IL-4 and IL-13 were decreased after secondary *E. acervulina* infection. The only notable changes following *E. tenella* infection were increased level of GM-CSF, and TGF-β4 and decreased levels of IL-4 and IL-13 (Fig. 3).

3.4. Chemokine response during Eimeria infection

Chemokines are important mediators of normal and inflammation-induced cell trafficking. Among the seven chicken chemokines analyzed, five were

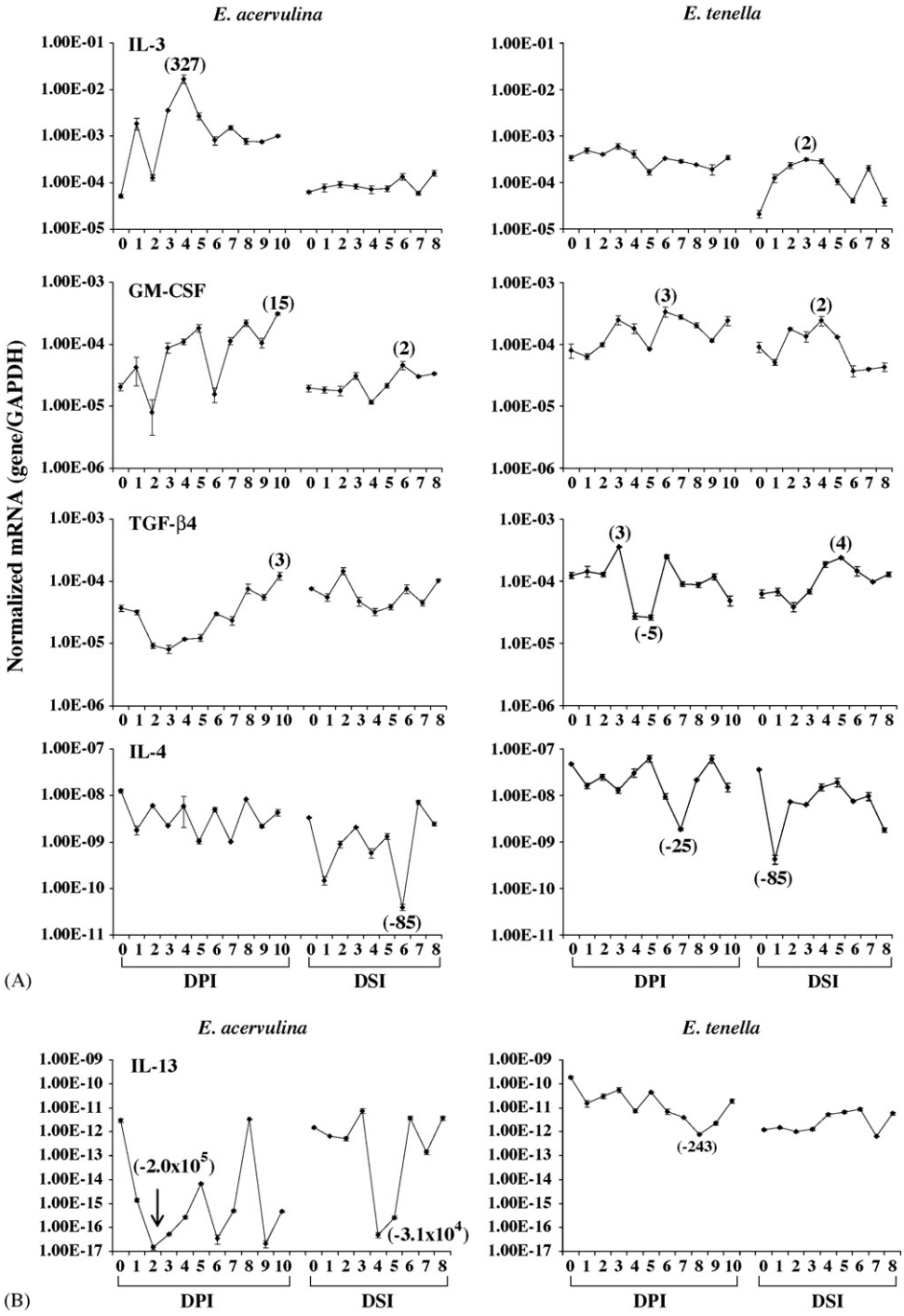

Fig. 3. Th2, anti-inflammatory (TGF-β4) and GM-CSF cytokine mRNA levels in intestinal IELs after *E. acervulina* or *E. tenella* primary and secondary infections. Chickens were infected with *E. acervulina* and *E. tenella* and mRNA levels determined as described in Fig. 1.

moderately up-regulated (6–170-fold) following primary *E. acervulina* or *E. tenella* infections (IL-8, lymphotactin, MIF, MIP-1β, and K203) (Fig. 4). Transcripts encoding SDF-1 were down-regulated after primary infection by either parasite. None of the mRNAs were substantially altered following secondary infections except for up-regulation of MIP-1β (4-fold) and K60 (6-fold) and down-regulation of SDF-1 (17-fold).

3.5. Cytokine and chemokine responses of IEL subpopulations during Eimeria infection

Next, we examined transcript levels of a subset of seven cytokines following *E. acervulina* or *E. tenella* primary infections in TCR1, TCR2, CD4, and CD8 depleted IEL subpopulations sorted by magnetic cell separation system. To confirm magnetic cell separation into marker-specific subpopulations, each negatively separated IEL subpopulation was processed for flow cytometric analysis. Cell separation effectively led to marker-specific depleted and enriched subpopulations with less than 2% positive for CD4, TCR1 and TCR2 depleted cells and about 8% for CD8 depleted cells (data not shown). This group of mRNAs (IFN-γ, IL-2, IL-3, IL-10, IL-15, IL-16, MIF), as well as that encoding T-bet, included those that have previously been correlated with protection against coccidiosis (Lillehoj and Lillehoj, 2000; Yun et al., 2000a). As shown in Fig. 5, transcripts for IFN-γ, IL-2, IL-3, IL-15, MIF, and T-bet were increased in $TCR2^+$ cells and that for IL-10 was elevated in $CD4^+$ cells following primary *E. acervulina* infection. No changes in $TCR1^+$ cells, and increased IL-2 and IL-16 in $CD8^+$ cells, were evident in *E. acervulina* infected animals. Following *E. tenella* infection, IL-16 was increased in $TCR1^+$ cells, IFN-γ,

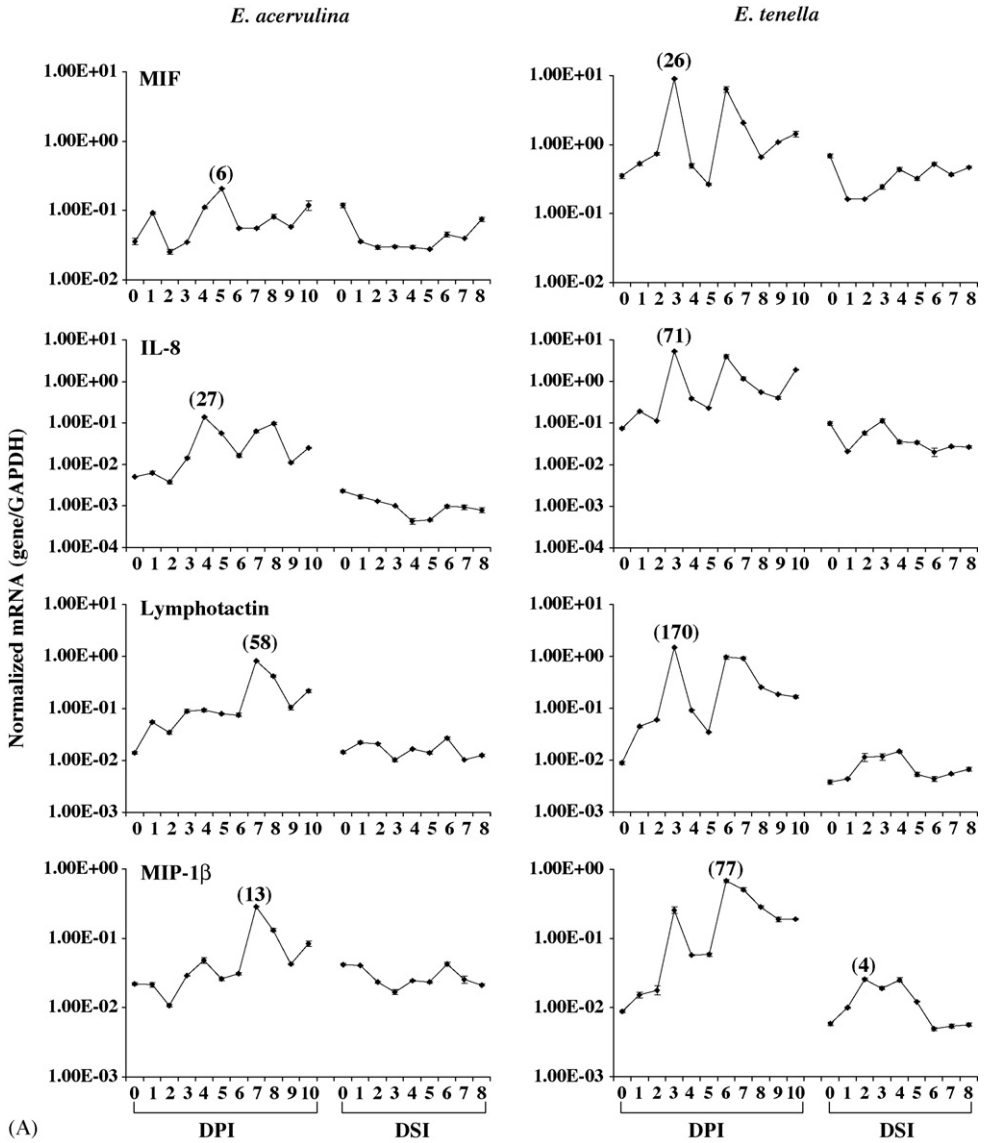

Fig. 4. Chemokine mRNA levels in intestinal IELs after *E. acervulina* or *E. tenella* primary and secondary infections. Chickens were infected with *E. acervulina* and *E. tenella* and mRNA levels determined as described in Fig. 1.

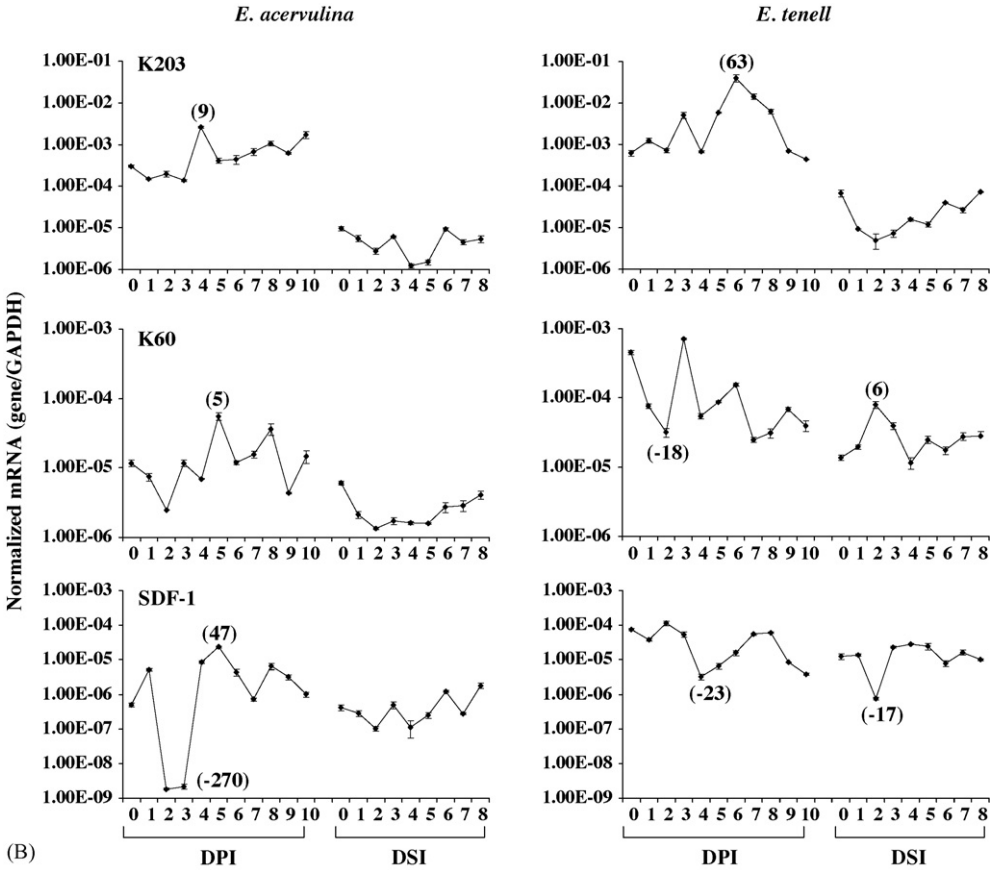

Fig. 4. (*Continued*).

IL-3, IL-10, and T-bet were elevated in $TCR2^+$ cells, and IFN-γ, IL-3, IL-15, and MIF were higher in $CD4^+$ cells. $CD8^+$ cells did not possess increased levels of any of the mRNAs examined.

4. Discussion

In this study, we investigated the kinetic response of immune-related cytokines and chemokines in intestinal IELs following experimental infections of chickens with *E. acervulina* or *E. tenella.* In general, cytokine/chemokine mRNA levels after primary infection with either parasite were highly up-regulated, whereas those following secondary infection were relatively unchanged. Furthermore, a correlation was found between cytokines previously identified as mediators of protection against coccidiosis and their transcript levels in $TCR2^+$ (for *E. acervulina*) or $CD4^+$ (for *E. tenella*) IELs.

IL-1β and IL-18 are structurally homologous proteins that play critical roles in initiating inflammation. Similar to the results reported here, IL-1β mRNA levels were observed to increase at post-primary infection with *E. maxima* and *E. tenella* respectively (Laurent et al., 2001) as well as in *Salmonella* infection (Withanage et al., 2004). Moreover, our recent study utilizing a chicken macrophage microarray also identified IL-1β and IL-18 as highly up-regulated gene transcripts during experimental coccidiosis (Dalloul et al., 2006). In mammals, IL-18 is an inducer of cell-mediated immunity, especially in combination with IL-12, and is primarily associated in Th1 responses to intracellular pathogen infections, such as coccidiosis (Biet et al., 2002; Dinarello and Fantuzzi, 2003). In chickens, heterophils from *Salmonella*-resistant chickens displayed significantly higher levels of IL-18 mRNA compared with heterophils from *Salmonella*-susceptible animals (Swaggerty et al., 2004). Thus, the increased levels of transcripts for several chicken pro-inflammatory cytokines that we observed suggest that these molecules may enhance protective immunity against *Eimeria* infection, and studies examining their potential as coccidiosis vaccine adjuvant are currently ongoing in our laboratory.

Intestinal inflammation observed in *Eimeria*-infected chickens is correlated with infiltration of macrophages and T lymphocytes and accompanied by increased production of cytokines and chemokines (Vervelde and Jeurissen, 1995). Interferons were first described in chickens (Isaacs and Lindenmann, 1957) and have been shown to have various immunomodulating effects on a wide variety of tissues. IFN-γ production during

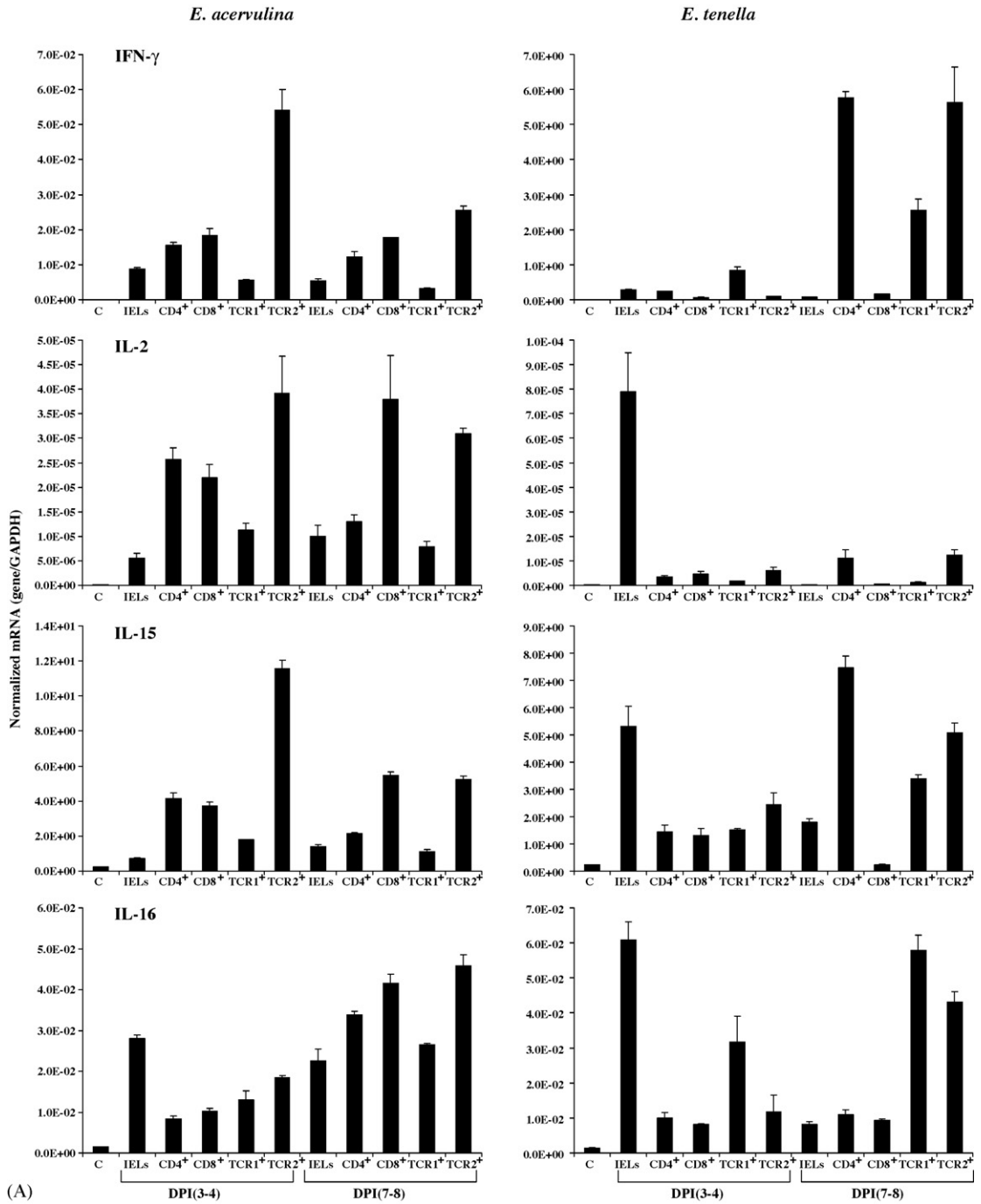

Fig. 5. Pro-inflammatory, Th1, and Th2 cytokine and chemokine mRNA levels in intestinal IEL subpopulations after *E. acervulina* or *E. tenella* primary and secondary infections. Chickens were infected with *E. acervulina* and *E. tenella*, IELs were separated into $TCR1^+$, $TCR2^+$, $CD4^+$, and $CD8^+$ subpopulations which were each depleted using marker-specific antibodies by magnetic BD IMag cell separation system at 3–4 DPI or 7–8 DPI, and mRNA levels determined as described in Fig. 1. C, non-infected IELs of 3 weeks age; IELs, non-sorted cells.

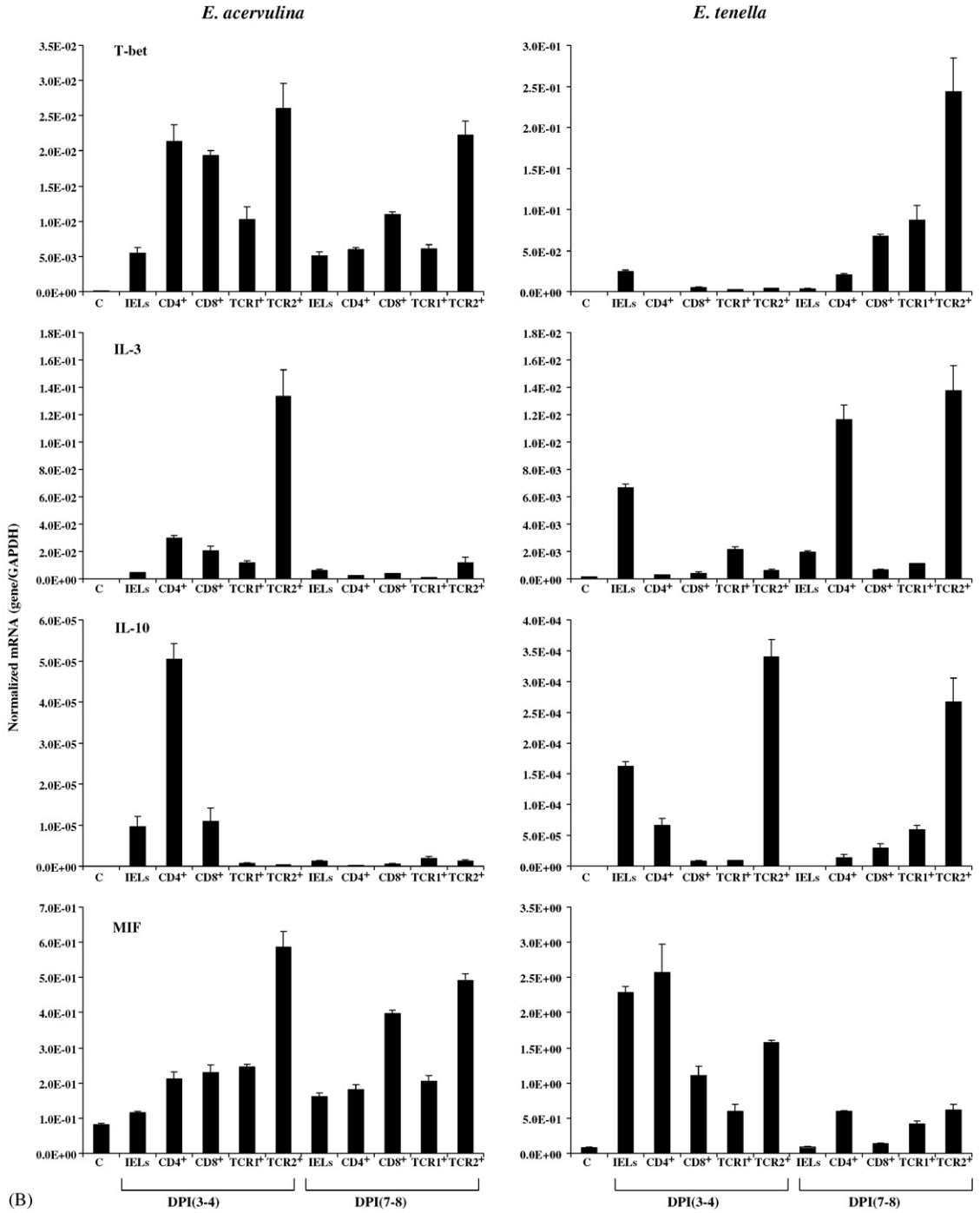

Fig. 5. (*Continued*).

coccidiosis has been examined by quantitative RT-PCR (Choi et al., 1999; Yun et al., 2000c) and more recently by gene expression profiling (Min et al., 2003). After *E. tenella* infection, IFN-γ mRNA expression was detected in lymphocytes isolated from the intestinal ceca of *E. tenella*-infected SC strain chickens, and the selective depletion of $CD4^+$, but not $CD8^+$ cells, reduced IFN-γ production (Yun et al., 2000a). Laurent et al. (2001) showed that IFN-γ expression in the cecum and jejunum of White Leghorn chickens increased more than 200-fold at day 7 post-primary infection with *E. tenella* and *E. maxima* as reported with *E. maxima* infection by

Rothwell et al. (2004). The current studies support these prior observations since we noted significant up-regulation of IFN-γ expression in the duodenum of *E. acervulina*-infected chickens and ceca of *E. tenella*-infected chickens, probably due in large part to the recruitment and stimulation of $TCR2^+$ and $CD4^+$ IELs, respectively. Because NO is produced by iNOS in activated macrophages in response to IFN-γ (MacMicking et al., 1997), the high levels of IFN-γ and iNOS transcripts in the intestinal mucosa of infected animals are probably directly related.

Like IL-1β and IL-18, IL-2 and IL-15 are also structurally homologous Th1 or Th1 related cytokines produced by mononuclear phagocytes and other cell types in response to viral or protozoan infection (Sundick and Gill-Dixon, 1997; Lillehoj et al., 2001). Both cytokines stimulate proliferation of chicken T lymphocytes and NK cells (Choi and Lillehoj, 2000; Lillehoj et al., 2001). In a previous report, a significant enhancement of IL-2 transcript levels was observed in the spleen and intestine following primary and secondary infections with *E. acervulina* (Yun et al., 2000a). Furthermore, the efficacy of an *Eimeria* experimental subunit vaccine against *E. acervulina* coccidiosis was markedly enhanced when it was administered in combination with the IL-2, IL-15, IL-16, or IL-18 Th1 or Th1 regulatory cytokines (Ding et al., 2004). These results are consistent with our current data. In contrast, after *E. tenella* infection the levels of IL-15 mRNAs remained relatively constant while those of IL-2 were markedly down-regulated. It remains to be determined if the difference between the cytokine responses induced by these two *Eimeria* species is related to their antigenicity, pathogenicity, and/or other unknown factors. Finally, IL-10 plays an important immunoregulatory role in the intestine, especially as a differentiation factor for a novel subset of T cells with suppressor function (Rothwell et al., 2004). IL-10 inhibits the synthesis of pro-inflammatory cytokines (including IL-1β, TNF-α, and IL-6), thus down-regulating inflammatory Th1 responses (de Waal Malefyt et al., 1991; Groux and Powrie, 1999). In chicken, IL-10 mRNA expression significantly increased after *E. maxima* infection (Rothwell et al., 2004). Similarly, our data indicate that IL-10 expression was moderately increased (<20-fold) following primary *E. acervulina* or *E. tenella* infection. Because chicken pro-inflammatory cytokines were also increased at this time, it suggests that the different cytokine activities may be compartmentalized to distinct subregions or cell types of the gut, or that chicken IL-10 activity may not reflect that of its mammalian counterpart. Th1 regulatory cytokines, iNOS, MyD88, TRAF-5, and T-bet also showed similar patterns with Th1 cytokines following infection with both *Eimeria* indicating a role in signal transduction during Th1 response as in mammals (Fig. 2).

Another set of molecules involved in the mucosal immune response are Th2 cytokines. In mammals, it has been known for some time that the balance between Th1/Th2 lymphocyte subsets determines susceptibility to some disease states. Thus, an unusually dominant Th1 response is often associated with autoimmunity, while improper development of Th2 immunity can lead to allergic diseases (Hwang et al., 2005). Chicken Th2 cells are necessary for inducing the humoral response to combat parasite invasion (Avery et al., 2004; Mowen and Glimcher, 2004; Degen et al., 2005). As in mammals, the chicken genome contains a cluster of Th2 cytokine genes containing IL-3, IL-4, IL-13, and GM-CSF, all of which are expressed in lymphoid tissues (Avery et al., 2004). Regulators of inflammation, TGF-β4 and GM-CSF are slightly increased in later days of primary *E. acervulina* infection or biphasic pattern in *E. tenella* (Fig. 3). It is associated with down-regulating inflammatory (Th1) response and results in drive Th2 cell development.

Especially chicken IL-3 was demonstrated at comparatively high levels in all tissues including cecal tonsil, which concurs with our expression data in *E. acervulina* primary infection, but not in *E. tenella.*

Chemokines are cytokines with chemotactic activity that act primarily by attracting leukocytes to sites of inflammation and facilitating their migration from the circulation into infected tissue to mediate host defense mechanisms (Ebnet and Vestweber, 1999). Chemokines are grouped into four subfamilies characterized by the position of their amino-terminal cysteine residues (Zlotnik and Yoshie, 2000) and about 23 chemokines were identified in chicken (Hwang et al., 2005; Kaiser et al., 2005; Wang et al., 2005; DeVries et al., 2006). In general, C and CC chemokines regulate the migration of monocytes, eosinophils, basophils, and T lymphocytes (Taub et al., 1993; Cook, 1996; Siveke and Hamann, 1998), whereas CXC chemokines recruit neutrophils (Laurent et al., 2001). Th1 and Th2 lymphocytes differ in their intrinsic migratory properties, which is mirrored in their chemotactic responsiveness to different chemokines. Thus, the CC chemokines MIP-1α, MIP-1β, and RANTES are efficient attractants for Th1 $CD4^+$ and macrophages or monocytes (Mantovani et al., 2003) and the CXC chemokine monocyte chemotactic protein-1 (MCP-1) and SDF-1α attract both Th1 and Th2 cells (Siveke and Hamann, 1998). Laurent et al. (2001) showed that the levels of mRNAs encoding MIP-1β and

K203 were up-regulated respectively in the ceca in response to *E. tenella* infection, and in the jejunum in response to *E. maxima* infection. Moreover, Withanage et al. (2004) reported that IL-8, K60, and MIP-1β levels were significantly upregulated in the intestinal tissues of infected birds with *Salmonella enterica* serova Typhimurium.

Similarly, we also observed that MIF, IL-8, Lymphotactin, MIP-1β, and K203 transcripts were up-regulated to a greater extent in *E. tenella* compared with *E. acervulina* primary infection. Especially, the expression patterns of CC chemokine K203, MIF, and C chemokine lymphotactin showed biphasic patterns at day 3 and day 6 or 7 following *E. tenella* primary infection, but not in *E. acervulina*. It might be cause of strong pathogenic hemorrhage of *E. tenella*. In summary, our results suggest that chicken chemokines play an important role in the regulation of local immunity in the gut by influencing the balance between T lymphocyte subpopulations (Siveke and Hamann, 1998).

In conclusion, the results presented in this report characterize the dynamics of chicken cytokine/chemokine responses associated with *E. acervulina* and *E. tenella* infection. During coccidiosis, both types of immune effecter molecules play key roles in protective immunity to parasite infection. It must be emphasized, however, that particular cytokine/chemokine transcripts may not correlate with protein expression, and current studies in our laboratory are directed at verifying these results at the protein level. If confirmed, these results will provide a rational basis for use of cytokines and chemokines as therapeutic agents against coccidiosis.

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